REMARKS

Prosecution

Applicants respectfully requests reconsideration of the outstanding rejections set forth in the Office Action mailed on November 18, 2005 in view of Applicants' instant claim amendments and the following Remarks.

Claim Amendments

Upon entry of the foregoing amendment, claims 1-2 and 4-19 are pending in the application. Claim 3 has been cancelled and the subject matter reiterated in new claims 18 and 19. Claims 1, 2, 4, 5, 12, and 13 have been amended. Applicants respectfully request entry of the above amendment and submits that the above amendment does not constitute new matter. Support for the amendments to the claims can be found throughout the specification and in the claims as originally filed. Support for the amendment to claim can be found, *inter alia*, in the specification, and in particular, at pages 5-6 and 24-25.

Restriction Requirement

Applicants maintain their traverse of the Restriction Requirement mailed on September 22, 2005 for the reasons as set forth in the Response to Restriction Requirement filed on October 21, 2005. Applicants respectfully request that all claims be rejoined and examined. Applicants reserve the right to pursue subject matter of non-elected claims in continuation, continuation-in-part, and divisional applications pursuant to 35 U.S.C. §§ 120 and 121.

Claim Objections

The Office Action objected to claims 2 and 4 because of typographical errors which have been corrected by Applicants.

Reconsideration and withdrawal of the objection is respectfully requested.

Enablement Rejection under 35 U.S.C. § 112 ¶ 1

The Office Action rejected claims 1-6, 16, and 17 under 35 U.S.C. § 112 ¶ 1 because the specification, while being enabled for *a method of proliferating cardiomyocytes in vitro by introducing adenoviral vectors expressing a D-type cyclin, CDK4, or CDK6 and a nuclear*

localization signal, does not reasonably provide enablement for *an in vitro or in vivo method of proliferating any terminal differentiated cell by introducing any cyclin and any cyclin dependent kinase into the cell.* Applicants respectfully traverse this rejection.

The Office Action asserts that the specification does not provide the structural and functional requirements to teach one of skill in the art to make and/or use the full scope of the claimed method, specifically that the specification does not support use of the method *in vitro* or *in vivo*; using any cyclin; and any cyclin dependent kinase into a cell.

The Office Action failed to meet the legal standard for establishing an enablement rejection because the Office Action did not establish a *prima facie* case that undue experimentation is required to make and use the instantly claimed invention. M.P.E.P. § 2164.01.

Additionally, the Office Action used the incorrect legal standard in determining enablement. The Office Action states that the examples in the specification are not sufficient to enable the claims. To the contrary, the Federal Circuit ruled in In re Brana, the USPTO is not the Food and Drug Administration ("FDA") and cannot require the same standards as the FDA. 51 F.3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995). In view of In re Brana, the examples in the specification are sufficient to enable the invention because the threshold for patentability, especially enablement, is far lower than that of clinical trials. Thus the holding in In re Brana established the rule that for meeting the requirements of 35 U.S.C. § 112 ¶ 1, a patent application need only use art-accepted models of a disease or therapy. Further, the Federal Circuit also held that to comply with the enablement requirement of 35 U.S.C. § 112 ¶ 1, it is not necessary to "enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect." CFMT, Inc. v. Yieldup Int'l Corp., 349 F.3d 1333, 1338, 68 USPQ2d 1940, 1944 (Fed. Cir. 2003). Therefore the instant application need only teach how to make and use the invention and not a, "perfected, commercially viable embodiment".

The Office Action purports to require Applicants to enable every aspect of gene therapy. The instant claims pertain to the use of specific genes, D-type cyclin in conjunction with CDK4 or CDK6 to stimulate mitogenesis in terminally differentiated cells such as cardiomyocytes or pancreatic cells and, in contrast to the Office Action's assertions, the specification need only

teach one method of effecting the claims and need not enable the full scope of the art. In re Fisher, 427 F.2d 833, 166 USPQ 18 (CCPA 1970).

In addition, the Office Action asserts that, "[t]he prior art did not compensate for the lack of guidance in the specification". This is the reverse of the legal standard. To be patentable, the specification solves problems present in the prior art. Instantly, the specification demonstrates the induction of cell division in cardiomyocytes *in vivo* using a gene therapy method. Specification at Example 4. Thus, it is not the prior art that needs to provide guidance to enable the claims but the specification. United States v. Telectronics, Inc., 857 F.2d 778, 8 USPQ2d 1217 (Fed. Cir. 1988).

On the nature of the invention and the breadth of the claim, Applicants respectfully notes that the claims require the cyclin to be a D-type cyclin and the cyclin-dependent kinase is CDK4 or CDK6, both of which are described as enabled in the Office Action. The specification provides sufficient disclosure on how to make and use a method of proliferating terminal differentiated cells comprising: introducing a D-type cyclin and a cyclin dependent kinase into the nucleus of terminal differentiated cells, and cultivating or holding said cells.

On the guidance in the specification/existence of a working example, the Applicants solved the problem of inducing proliferation in cardiomyocytes without triggering apoptosis by introducing a cyclin D1 gene with a nuclear localization signal (NLS) and a CDK4 gene via an adenovirus vector into cultured cardiomyocytes. This lead not only to expression of the genes but proliferation of the cardiomyocytes. Specification at 5. The specification goes further by providing an example were rats were treated with recombinant adenovirus carrying D1NLS/CDK4 or the *lacZ* gene shows the expression of these genes demonstrating that the instant vehicle works *in vivo*. Figure 11; Example 5. At page 25 of the specification, the Applicants teach that infection of an adenovirus vehicle comprising D1NLS and another comprising CDK4 lead to the proliferation of cardiomyocytes *in vivo* as evidenced by the expression of Ki-67 nuclear protein which is only expressed in proliferating cells in all phases of the cell cycle. In the specification, Figure 11 shows the visualized views of expressed proteins by immunoflourescence staining of heart tissue section after infection of recombinant adenovirus comprising D1NLS/CDK4 or lacZ gene in rat cardiomyocytes *in vivo*. See also Tamamori-Adachi *et al.* (January 10, 2003) "Critical role of cyclin D1 nuclear import in cardiomyocyte proliferation." Circ Res. **92**(1): 12-9. As this is an art-accepted model, Applicants have met their

burden of enablement in view of In re Brana. As discussed above, the specification need not demonstrate every aspect of the invention but only show how to make and use the invention, the claims are enabled.[1]

On the state of the art/predictability of the art, the Office Action cites Pagano & Jackson (September 3, 2004) "Wagging the dogma; tissue-specific cell cycle control in the mouse embryo." Cell **118**(5): 535-8, a post-filing reference, that teaches that D-type cyclins activate CDK4 or CDK6. Id. at 535. Applicants note that use of a post-filing reference is generally improper unless it is evidence that the disclosed invention was not possible at the time of filing. In re Wright, 999 F.2d 1557, 27 USPQ2d 1510, 1513-14 (Fed. Cir. 1993). However, in the instant application, the Pagano & Jackson reference support the claims and as such do not provide evidence of unpredictability.

The Office Action cites Brooks & La Thangue (October 10, 1999) "The cell cycle and drug discovery: the promise and the hope." Drug Discovery Today **4**(10): 455-464 which discusses cell cycle proteins and their potential role in tumor genesis. The Brooks *et al.* reference does not provide evidence of unpredictability and, instead, provides evidence that the state of the art favored the claims because of the role cyclins, particularly, D-type cyclins, play in the cell cycle. The Office Action asserts that cyclins and CDKs may result in deregulation of the cell cycle that can lead to tumorigenesis or hyperfroliferation of cells, but adenoviruses, one of the vehicles taught by the specification, generally do not stably integrate the transgene into the host genome. This property avoids the concern raised by the Office Action that the gene delivery vehicle would not cause tumorgenesis because integration of the transgene is usually the cause of the tumorogeneis if seen in gene therapy. Adenovirsus also show effective gene expression in both proliferating and non-proliferating cells, avoiding another concern raised by the Office Action. Rubayni at 118.

The Office Action cites Verma & Somia (September 18, 1997) "Gene therapy—promises, problems and prospects." Nature **389**: 239-242 which teaches the properties of an ideal vector for use in gene therapy methods. Id. at 241. In essence, Verma & Somia provide guidance on how to overcome the obstacles existing prior to the filing of the instant application. The teachings of the instant application in conjunction with Verma & Somia provides adequate

[1] This is the "proof of principle" discussed by Ross *et al.*

guidance to practice the invention as instantly claimed. Also, the authors express their view that, "in the not too distant future, gene therapy will become as routine a practice as heart transplants are today." Id. at 242. Thus, Verma & Somia supports the specification, filed 4 years after it was published, as enabling.

The Office Action cites Marshall (August 25, 1995) "Gene Therapy's Growing Pains." Science **269**: 1050-1055 which is an opinion article from 1995 that critiques Congress and the NIH. This article does not address the state of the art nor provide useful information on the technology as it existed in 1995. In addition, the article by Marshall is mostly concerned with the ethical and political dimensions of gene therapy which is outside the purview of the USPTO. Juicy Whip Inc. v. Orange Bang Inc., 382 F.3d 1367, 72 USPQ2d 1385 (Fed. Cir. 2004).

The Office Action cites Eck *et al.* Chapter 5: "Gene-Based Therapy" Goodman & Gilman's The Pharmacological Basis of Therapeutics (9th Ed.) which provides a comprehensive review of the state of the art of gene therapy in 1996. Based on the disclosure of Eck *et al.* the skilled artisan had a solid basis upon which to make and use the instantly claimed invention. Eck *et al.*, contrary to the Office Action, is optimistic and provides ample guidance to make and use viral and non-viral gene therapy vectors including a Table of NIH approved gene therapy trials. Id. at Table 5-1.

Applicants respectfully note that the Office Action cited references which were 4-5 years older than the application. In particular, Verma & Somia antedate the application by four years, Eck *et al.* (1996) and Ross *et al.* both antedate the application by 5 years, and Marshall is a news article published 6 years prior to the filing of the application. Contrary to this position, the state of the prior art is what one skilled in the art would have known, at the time the application was filed, about the subject matter to which the claimed invention pertains. And the relative skill of those in the art refers to the skill of those in the art in relation to the subject matter to which the claimed invention pertains at the time the application was filed. M.P.E.P. § 2164.05(b). In patent law, the state of the art for a given technology is not static in time, especially in a rapidly developing art such as molecular biology. For instance, it is entirely possible that a disclosure filed on January 2, 1996, may not have been enabled. However, if the same disclosure had been filed on January 2, 2000, it could enable the claims because of progress made in the art. The state of the prior art provides evidence for the degree of predictability in the art and is related to the amount of direction or guidance needed in the specification as filed to meet the enablement

requirement. M.P.E.P. § 2164.05(a). Therefore, in an Office Action, the USPTO must evaluate the state of the prior art for an application based on its filing date. M.P.E.P. § 2164.05(a).

Furthermore, the enablement requirement of 35 U.S.C. § 112 ¶ 1 requires the specification to be enabling only to a person "skilled in the art to which it pertains, or with which it is most nearly connected." In general, pertinent art is defined in terms of the specific problem to be solved rather than in terms of the technology area for which the invention is used. M.P.E.P. § 2164.05(a).

In particular, the Office Action asserts that gene therapy methods were not routine and had difficulties with vector selection, delivery mode, and expression. The instant invention does not require the "swapping out" of a defective gene with a functional gene. All that is required is that the D-type cyclin be introduced into a terminally differentiated cell and induce mitosis. This is a lesser hurdle to overcome than the other gene therapy trials detailed by Ross *et al.* and Rubanyi. Rubanyi teaches that this has been accomplished by viral systems in human cells. Id. at 116.

The Office Action bases its assertions on 5-6 year old references the expectation of success was "extremely low". This is in contrast to Rubanyi which teaches that Rubanyi (2001) "The future of human gene therapy." Molecular Aspects of Medicine **22**: 113-142 teaches that successful gene therapy requires therapeutically suitable gene, appropriate gene delivery system, proof of principle of efficacy and safety in appropriate preclinical models and suitable manufacturing and analytical processes to provide well-defined gene therapy products for use. Further, Rubanyi teaches that,

> "[t]he most promising areas for gene therapy today are hemophilias, for monogenetic diseases, and cardiovascular diseases...among multigeneic diseases. This is based on the relative ease of access of blood vessels for HGT, and also because existing gene delivery technologies may be sufficient to achieve effective and safe therapeutic benefits for some of these indications (transient gene expression in some but not all affected cells is required to achieve a therapeutic effect at relatively low [safe] dose of vectors)." Id. at 113-114.

Contrary to the position of the Office Action, Rubanyi actually supports an embodiment of the invention as enabled because of the relative ease of access to the target and the fact that existing gene delivery technology are sufficient to achieve effective expression. Also, Rubanyi states that transient gene expression in some but not all of the cells in the target is sufficient to yield a therapeutic effect. Adenoviruses, one of the vehicles taught by the specification, can be

produced in high titre. This property overcomes one of the concerns raised by the Office Action. Adenovirsus also show effective gene expression in both proliferating and non-proliferating cells. Id. at 118. Further, Rubanyi teaches that success has already been seen in models of cardiovascular disease using cyclin and cyclin-dependent kinase inhibitors. Id. at 129.

The Office Action relies upon Juengst (2003) "What next for human gene therapy?" British Medical Journal **326**: 1410-11 is an opinion article. The M.P.E.P. states that opinion is given no weight. Id. § 2164.05. Further the example cited by the Office Action to illustrate the "unpredictable nature of gene therapy" is not analogous to the instant claims. In that case, the gene therapy vector was administered *ex vivo* to lymphocytes and reintroduced into the patient. Two of nine patients in a study developed leukemia because the transgene inserted at or near the LMO-2 gene. Here the subpopulation is selected, terminally differentiated cells, and the target gene is known, D-type cyclins with a NLS and CDK4 or CDK6 and only requires transient expression to have a therapeutic effect. Further, a single example of complications in a clinical trial does not carry enough weight to declare an entire field of endeavor as "unpredictable". This is especially pertinent in view of the working examples presented in the specification. In addition, the article by Juengst is mostly concerned with the ethical dimensions of clinical trials for gene therapy which is outside the purview of the USPTO. Juicy Whip Inc. v. Orange Bang Inc., 382 F.3d 1367, 72 USPQ2d 1385 (CA FC 2004).

On the quantity of experimentation, the Office Action asserts that a large amount of experimentation would be necessary to determine the cyclin and the CKD that would be able to promote proliferation of every terminally differentiated cell type either *in vitro* or *in vivo*. The Office Action asserts that a "large amount of experimentation would be necessary" to determine the cyclin and CDK that would be able to promote proliferation in terminally differentiated cell types both *n vitro* and *in vivo*. Applicants respectfully traverse because the claims are drawn to D-type cyclins and CDK4 and CDK6 both of which have known and proven effects to stimulate mitogeneiss as members of the cell cycle. Further, the claims only requires that the terminally differentiated cells divide in response to D-type cyclin and CDK which has been demonstrated by the specification. The Office Action asserts that "a large amount of trial and error experimentation" is necessary to practice the invention. As cited by the Office Action, the standard for enablement is not "large amount" but an "undue amount of experimentation" and in

view of the guidance present in the specification the skilled artisan can readily make and use the invention as claimed.

Rubanyi also teaches that "promising therapeutic effects" have been observed in models using cyclin or cyclin-dependent kinases. Thus in contrast to the Office Action's position, Rubanyi provides discussion of how use of gene therapy to treat cardiovascular gene therapy is a readily applicable method and only is disparaging of more general, ill-defined gene therapy regiments which lack a specific gene target or a specific tissue target. Id. at Table 6. This is in contrast with the instant claims which are drawn to a specific gene (D-type cyclins) and a specific tissue target (terminally differentiated cells).

In the instant case, the claims are directed to D-type cyclin and its role in terminally differentiated cells. It is undisputed that D-type cyclins play a role in cell cycle and when activated cause cells to grow and divide. Therefore D-type cyclins are a therapeutically suitable gene. The second requirement taught by Rubanyi is an appropriate gene delivery system and in the instant case the prior art teaches several, as discussed by Ross *et al.* at Table 2, and in the specification which teaches that adenovirus, adeno associated virus, retrovirus, vaccinia virus, chick poxvirus, and papovavirus may be used as vehicles in the instant invention. Specification at 11. The specification provides ample guidance on how to make gene delivery vehicles. Speciation at 12-13; Examples 1-2. Instantly, the claims are limited to specific gene target in a subset of cell populations avoiding may of the problems associated with gene therapy. Here, the Applicants are targeting a known and well-characterized genes, D-type cyclins, with a fully supported target regiment to trigger a specific cellular pathway. This is in contrast to the references cited by the Office Action which details a litany of problems with undefined targets and broad targets.

Therefore, a person of skill in the art has sufficient guidance from the specification in the form of the examples, detailed activities and methods for testing for them and a person of skill in the art could make and use the method encompassed by the claims with nothing more than routine experimentation.

Reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

In view of the foregoing Amendment and Reply to the Office Action of November 18, 2005, Applicants respectfully submits that claims 1-2, 4-6, and 16-19 are in condition for allowance, and such disposition is earnestly solicited. Further, Applicants respectfully requests rejoinder of claims 7-15 and allowance of the claim 1-19. Should the Examiner believe that any patentability issues remain after consideration of this Response, the Examiner is invited to contact the Applicant's undersigned representative to discuss and resolve such issues.

In the event that a variance exists between the amount tendered and that deemed necessary by the U.S. Patent and Trademark Office to enter and consider this Response or to maintain the present application pending, please credit or charge such variance to the undersigned's **Deposit Account No. 50-0206**.

Respectfully submitted,

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APPENDIX A

Tamamori-Adachi *et al.* (January 10, 2003)
"Critical role of cyclin D1 nuclear import in cardiomyocyte proliferation."
Circ Res. **92**(1): 12-9

UltraRapid Communication

Critical Role of Cyclin D1 Nuclear Import in Cardiomyocyte Proliferation

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Abstract—Mammalian cardiomyocytes irreversibly lose their capacity to proliferate soon after birth, yet the underlying mechanisms have been unclear. Cyclin D1 and its partner, cyclin-dependent kinase 4 (CDK4), are important for promoting the G1-to-S phase progression via phosphorylation of the retinoblastoma (Rb) protein. Mitogenic stimulation induces hypertrophic cell growth and upregulates expression of cyclin D1 in postmitotic cardiomyocytes. In the present study, we show that, in neonatal rat cardiomyocytes, D-type cyclins and CDK4 were predominantly cytoplasmic, whereas Rb remained in an underphosphorylated state. Ectopically expressed cyclin D1 localized in the nucleus of fetal but not neonatal cardiomyocytes. To target cyclin D1 to the nucleus efficiently, we constructed a variant of cyclin D1 (D1NLS), which directly linked to nuclear localization signals (NLSs). Coinfection of recombinant adenoviruses expressing D1NLS and CDK4 induced Rb phosphorylation and CDK2 kinase activity. Furthermore, D1NLS/CDK4 was sufficient to promote the reentry into the cell cycle, leading to cell division. The number of cardiomyocytes coinfected with these viruses increased 3-fold 5 days after infection. Finally, D1NLS/CDK4 promoted cell cycle reentry of cardiomyocytes in adult hearts injected with these viruses, evaluated by the expression of Ki-67, which is expressed in proliferating cells in all phases of the cell cycle, and BrdU incorporation. Thus, postmitotic cardiomyocytes have the potential to proliferate provided that cyclin D1/CDK4 accumulate in the nucleus, and the prevention of their nuclear import plays a critical role as a physical barrier to prevent cardiomyocyte proliferation. Our results provide new insights into the development of therapeutics strategies to induce regeneration of cardiomyocytes. The full text of this article is available at http://www.circresaha.org. **(*Circ Res*. 2003;92:e12-e19.)**

Key Words: cardiomyocyte ■ cyclin D1 ■ CDK4 ■ nuclear localizing signals ■ cell cycle progression

Mammalian cardiomyocytes irreversibly withdraw from the cell cycle soon after birth.[1,2] Recent studies, however, have shown that the adult heart contains a small population of cardiomyocytes that retain the proliferative capacity.[3,4] For instance, a significant number (0.08%) of mitotic cardiomyocytes has been identified in regions adjacent to the infarcts.[4] However, cardiomyocytes remaining in these regions cannot reconstitute damaged tissue, at least in part, due to the quite limited proliferative capacity of cardiomyocytes. Therefore, cardiomyocyte loss is irreversible and frequently leads to diminished cardiac function and severe heart failure. Recent studies have demonstrated that the engraftment of cells derived from embryonic or bone marrow stem cells may be useful for regenerating the functional myocyte mass in the adult heart.[5–7] In addition to these approaches, evidence that the adult heart has limited but significant proliferative capacity raises the alternative possibility that increasing the number of the remaining cardiomyocytes by activating their proliferative potential could replace damaged myocardium. In this regard, investigating the inhibitory mechanism of cardiomyocyte proliferation might open new avenues of therapy with which to treat cardiovascular diseases that cause cardiomyocyte loss.

Progression of mammalian cell cycle is regulated by a family of cyclins and cyclin-dependent kinases (CDKs). During G1 phase, cyclin D1 and other D-type cyclins (D2 and D3) accumulate in response to mitogenic stimulation and assemble with their catalytic partners, CDK4 and CDK6. Cyclin D1 plays an important role for promoting G1-to-S phase progression by inactivating the action of the retinoblastoma protein (Rb) through phosphorylation. In addition to Rb phosphorylation, cyclin D/CDK complexes sequester CDK inhibitors such as $p21^{Cip1}$ and $p27^{Kip1}$ and thereby facilitate activation of the cyclin E/CDK2 and cyclin A/CDK2 com-

Original received June 19, 2002; resubmission received September 26, 2002; revised resubmission received November 1, 2002; accepted November 12, 2002.

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Circulation Research is available at http://www.circresaha.org

DOI: 10.1161/01.RES.0000049105.15329.1C

plexes required for entry into and the progression of S phase.[8,9] During cardiomyocyte differentiation, Rb phosphorylation and CDK activity are reduced in association with cell cycle arrest.[10] However, postmitotic cardiomyocytes still retain the capacity to respond to mitogenic stimulation, which induces hypertrophic cell growth and upregulates expression of cyclins and CDKs, including cyclin D1 and CDK4.[11,12] Thus, the mechanisms underlying cardiomyocyte withdrawal from the cell cycle have remained obscure. In this study, we studied the regulation of subcellular localization of cyclin D1 in neonatal cardiomyocytes. The results showed that the nuclear import of cyclin D1/CDK4 plays an important role in cardiomyocyte proliferation in vitro and in vivo.

Materials and Methods

Cell Culture

Cardiomyocytes were cultured as previously described[13,14] with some modifications. Heart ventricles were isolated from 17-day fetal or 3-day-old postnatal Sprague-Dawley rats, trisected, and then digested 4 times with collagenase type II (1 mg/mL, Worthington) in Ads buffer (in mmol/L: 116 NaCl, 20 HEPES, 1 NaH_2PO_4, 5.5 glucose, 5.4 KCl, 0.8 $MgSO_4$; pH 7.35) at 37°C for 20 minutes. The dispersed cells from each digestion were combined, washed, and then were purified by centrifugation through a discontinuous Percoll gradient of 1.050, 1.060, and 1.082 g/mL, respectively. The cells at the 1.060/1.082 interface were collected and used for cardiomyocyte cultures. The purified cardiomyocytes were plated on 60-mm dishes (2×10^6 cells per dish) or 25-mm collagen-coated coverslips in 6-well plates (2×10^5 cells per well) in minimum essential medium (MEM) supplemented with 5% calf serum (CS), penicillin (100 U/mL, GIBCO), and streptomycin (100 μg/mL, GIBCO). Cardiomyocyte cultures were incubated in serum-containing medium at 37°C for 24 hours in humidified air with 5% carbon dioxide, after which the medium was changed to serum-free MEM and incubated for another 24 hours. The purity of the cultures was determined by immunohistochemistry with anti-sarcomeric actin (M0874, DAKO) and FITC-conjugated anti-mouse antibodies (23799, Polysciences) 48 hours after plating. Only cultures that consist of >95% FITC-positive cells determined by counting 300 cells in 3 different fields were subjected to various analyses. Control REF52 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal calf serum (FCS).

Adenoviruses

Ad-Ras61L and Ad-GSKDN (gifts from J.R. Nevins, Duke University, Durham, NC),[15] Ad-D1 (a gift from J.H. Albrecht, Hennepin County Medical Center, Minneapolis, Minn),[16] Ad-LacZ,[17] and Ad-p16 and Ad-p21[12] have been described. To generate Ad-CDK4, a blunt-ended fragment encoding CDK4 was isolated from pCMV-CDK4 (a gift from S. van den Heuvel, Massachusetts General Hospital Cancer Center, Boston, Mass) and then cloned into the *SwaI* site of the cosmid pAxCAwt (Takara). To generate Ad-D1NLS, the fragment encoding mouse cyclin D1 was first cloned into pEF1 myc/nuc (Invitrogen) to fuse with NLSs (fusion to myc epitope and NLSs added ≈5 kDa to the gene product). The resultant fragment encoding D1NLS of the resulting plasmid was blunt-ended and cloned into the same site of pAxCAwt. Recombinant adenoviruses were generated by in vitro homologous recombination in 293 cells using the Adenovirus Expression Vector Kit (Takara) according to the manufacturer's instructions. Viruses were propagated in 293 cells, and virus stocks were prepared as described.[18] Viral titers were determined by an indirect immunofluorescent assay by using anti-72K serum.[18] Cells were infected at the indicated multiplicity of infection (MOI) in serum-free MEM for 60 minutes with brief agitation every 15 minutes. After infection, the medium was replaced with culture medium.

Western Blotting

Whole cell, cytoplasmic, and nuclear extracts were prepared as described.[19] Extracts prepared from equal number of cells were denatured and separated by 6% or 11% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore). The blots were probed with primary antibodies and bands were detected using horseradish peroxidase conjugated secondary antibodies (Amersham Pharmacia Biotech) and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The following antibodies were used in this study: mouse monoclonal anti-cyclin D1 (Ab-3, Oncogene science), anti-Rb (14001A, PharMingen), anti-sarcomeric actin (M0874, DAKO), anti-PCNA (sc-056), and anti-BrdU (Roche) antibodies, and rabbit polyclonal anti-Ki-67 (PRO229, YLEM), anti-β-galactosidase (β-gal) (ICN), anti-cyclin D2 (sc-593), anti-cyclin D3 (sc-182), anti-cyclin A (sc-751), anti-cyclin E (sc-481), anti-p21 (sc-6246), anti-CDK4 (sc-260), and anti-CDK2 (sc-163) antibodies (Santa Cruz Biotechnology).

Immunoprecipitation and Kinase Assays

Whole cell extracts were immunoprecipitated with anti-CDK4 (sc-260) followed by Western blotting with anti-cyclin D1 (Ab-3) or anti-p21 antibodies. To assay CDK2 kinase activity, extracts were immunoprecipitated with anti-CDK2 antibody and then histone H1 kinase was assayed as described.[11]

Immunofluorescent Staining and Cell Cycle Analysis

To examine the subcellular localization of cyclin D1, cells plated on glass coverslips were fixed in 70% ethanol, double-stained with anti-cyclin D1 and anti-sarcomeric actin antibodies, and then immunostains were visualized using fluorescent tyramide reagent according to the manufacturer's protocols (TSA-direct NEL-701, NEN). To analyze the cell cycle profile, fixed cells were stained with anti-sarcomeric actin and FITC-conjugated anti-mouse antibodies, followed by propidium iodide (50 μg/mL) containing RNase A (500 μg/mL). To determine the DNA content, the intensity of propidium iodide staining was analyzed on cell populations that were positive for FITC staining using a laser scanning cytometer (LSC 101) (Olympus). Confocal images were acquired using a confocal microscope (FV500) (Olympus).

In Vivo Study

All animal studies were in accordance with TMDU institutional guidelines. Apical injections of viruses into the myocardium were performed under direct visualization after thoracotomy of Wister rats (250 to 300 g). Virus mixture containing 1×10^{10} PFU of either Ad-D1NLS or Ad-D1, together with Ad-CDK4 (1×10^{10} PFU), was injected into hearts. To identify the infected area, Ad-LacZ (2×10^9 PFU) encoding β-galactosidase was added into the virus mixture. For controls, Ad-LacZ (2×10^{10} PFU) was used. In some experiments, intraperitoneal injections of 5-bromo-2′-deoxyuridine (BrdU) (50 mg/kg, Roche) were given every 12 hours before euthanasia to detect DNA synthesis. Four days after injections, hearts were fixed with 4% paraformaldehyde by perfusion. The sections of tissues were probed with anti-Ki-67 and anti-β-gal antibodies. To confirm viability of Ki67 positive cardiomyocytes, sections were subjected to terminal transferase-mediated dUTP nick end labeling (TUNEL) assay using the in situ Apoptosis Detection Kit (Takara) and then stained with anti-Ki67 antibody and 1 μg/mL of DAPI. For analysis of BrdU incorporation, fixed sections were denatured by incubation in 1 mol/L hydrochloric acid at 65°C for 30 minutes[20] and then probed with anti-BrdU (Roche) and anti-β-gal (ICN) antibodies. For detection of primary antibodies, sections were incubated with the appropriate secondary antibody mix containing anti-mouse and anti-rabbit antibodies conjugated with Alexa 488, Alexa 568 (Molecular Probes), or Ci5 (Jackson) for 1 hour at room temperature. Lastly, to identify cardiomyocytes, all sections were stained with anti-sarcomeric actin antibody covalently labeled with Alexa 488 or Alexa 555 using the Zenon One Mouse IgG1 Labeling Kit (Molecular Probes). Images were obtained

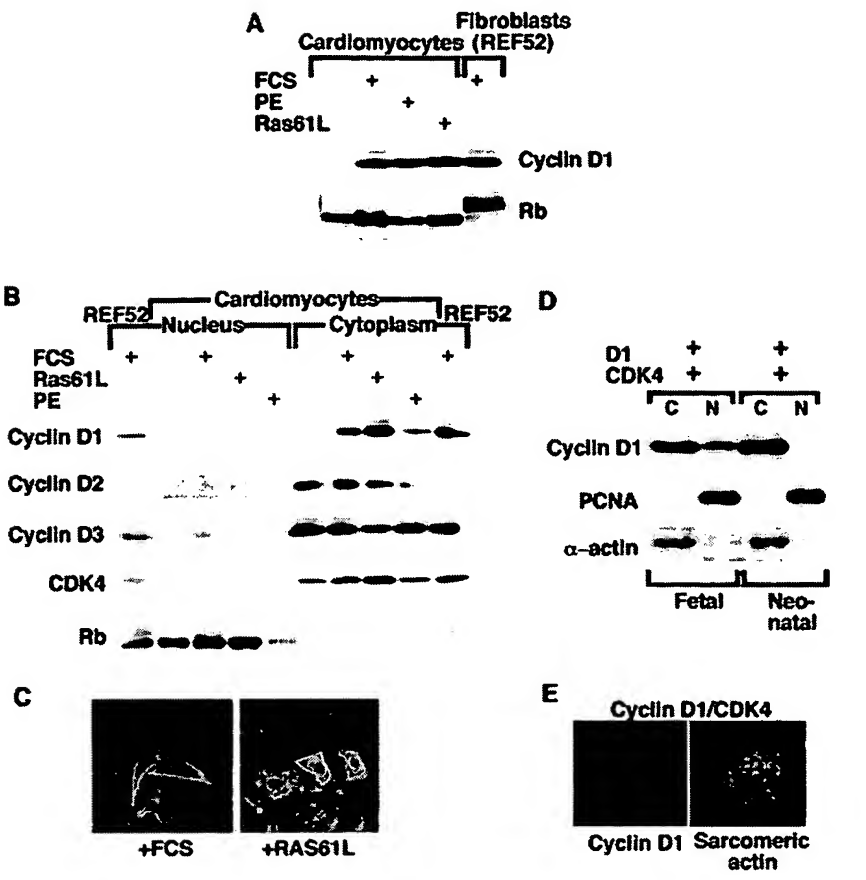

Figure 1. D-type cyclins and CDK4 localize in the cytoplasm in cardiomyocytes. Serum-starved cardiomyocytes were stimulated by 10% FCS or phenylephrine (10^{-6} mol/L) (PE), or infected with Ad-Ras61L at multiplicity of infection (MOI) of 100 focus forming units (FFU) per cell, as indicated. Cells were harvested 18 hours after FCS or PE addition, or 36 hours after infection. Cardiomyocytes cultured in media with 5% CS were infected with Ad-cyclin D1/Ad-CDK4 at MOI of 100 FFU/cell and harvested 48 hours after infection. Asynchronously growing REF52 cells were used as controls. A, Mitogenic or hypertrophic stimuli induce cyclin D1 expression but not Rb phosphorylation in cardiomyocytes. Whole cell extracts were assessed for Western blotting using anti-cyclin D1 and Rb antibodies. B, Cytoplasmic localization of D-type cyclins and CDK4 in cardiomyocytes. Nuclear and cytoplasmic extracts were analyzed as in A, by using the indicated antibodies. C, Cyclin D1 localizes in the cytoplasm in stimulated cardiomyocytes. Cells plated on glass coverslips were stimulated by FCS or Ras61L and stained with anti-cyclin D1 antibody. Immunostains were visualized using fluorescent tyramide reagent (NEN). D, Ectopic expressed wild-type cyclin D1 enter the nucleus in fetal cardiomyocytes (embryonic day 17), but not in neonatal cardiomyocytes. Nuclear and cytoplasmic extracts were assessed by Western blotting using anti-cyclin D1, anti-sarcomeric α-actin, and PCNA antibodies. E, Ectopic expressed wild-type cyclin D1 cannot localize in the nucleus in cardiomyocytes. Double immunofluorescent staining with anti-cyclin D1 (red) and anti-sarcomeric actin (green) as in C.

with a confocal laser microscope (FV500) (Olympus) or the laser-scanning confocal image system (Zeiss LSM510).

Results

We first examined the effects of mitogenic or hypertrophic stimuli on cyclin D1 expression and Rb phosphorylation in neonatal rat cardiomyocytes. Serum-starved cardiomyocytes isolated from 3-day old rats were stimulated with fetal calf serum (FCS) or phenylephrine (PE), an α-adrenergic receptor agonist, or infected with an adenovirus encoding activated ras (Ras61L), all of which induce hypertrophy in cardiomyocytes.[12,21–23] Western blotting of whole cell extracts revealed that all of these stimuli induced cyclin D1 protein in cardiomyocytes but not the Rb phosphorylation apparent in control proliferating REF52 cells (Figure 1A).

The subcellular localization of cyclin D1 is regulated during the cell cycle.[24–28] We next examined whether the subcellular localization of cyclin D1 might contribute to its inability to phosphorylate Rb in neonatal cardiomyocytes. Nuclear and cytoplasmic extracts from cells stimulated as described above were analyzed by Western blotting and immunostaining. The vast majority of cyclin D1 and CDK4, as well as other D-type cyclins (D2 and D3), localized to the cytoplasm, whereas Rb predominantly localized in the nucleus (Figures 1B and 1C). Moreover, even when cyclin D1 and CDK4 were overexpressed using recombinant adenoviruses in neonatal cardiomyocytes cultured in the presence of 5% calf serum (CS), exogenous cyclin D1 did not appear to accumulate in the nucleus (Figures 1D and 1E). In contrast, exogenous cyclin D1 accumulated in the nucleus in proliferating cardiomyocytes isolated from 17-day-old fetal hearts (Figure 1D). Finally, separation of nuclear and cytoplasmic fractions was confirmed by localization of proliferating-cell nuclear antigen (PCNA) and sarcomeric α-actin, which were predominantly detected in the nucleus and cytoplasm, respectively. Thus, the inability of cyclin D1 and CDK4 to phosphorylate Rb may be due to their cytoplasmic sequestration in cardiomyocytes.

We next examined whether nuclear targeting of cyclin D1 and CDK4 could induce the phosphorylation of endogenous Rb, thereby activating downstream events that are regulated by Rb in cardiomyocytes. To efficiently target cyclin D1 to the nucleus, we constructed an adenovirus encoding a variant of cyclin D1 (D1NLS) in which the C-terminal end of cyclin D1 was fused with triplicate monopartitie NLS derived from the SV40 large T antigen. Overexpression of D1NLS and CDK4 by the recombinant adenoviruses revealed that D1NLS protein accumulated in the nucleus, whereas exogenous wild-type cyclin D1 predominantly localized in the cytoplasm, even though these proteins were expressed at comparable levels (Figures 2A and 2B). Furthermore, D1NLS expression caused significant accumulation of CDK4 protein in the nucleus (Figures 2C, lane 4, bottom). To address the complex formation, nuclear and cytoplasmic extracts (used in Figure 2B) were immunoprecipitated by anti-CDK4 antibody followed by Western blotting with cyclin D1 antibody. Figure

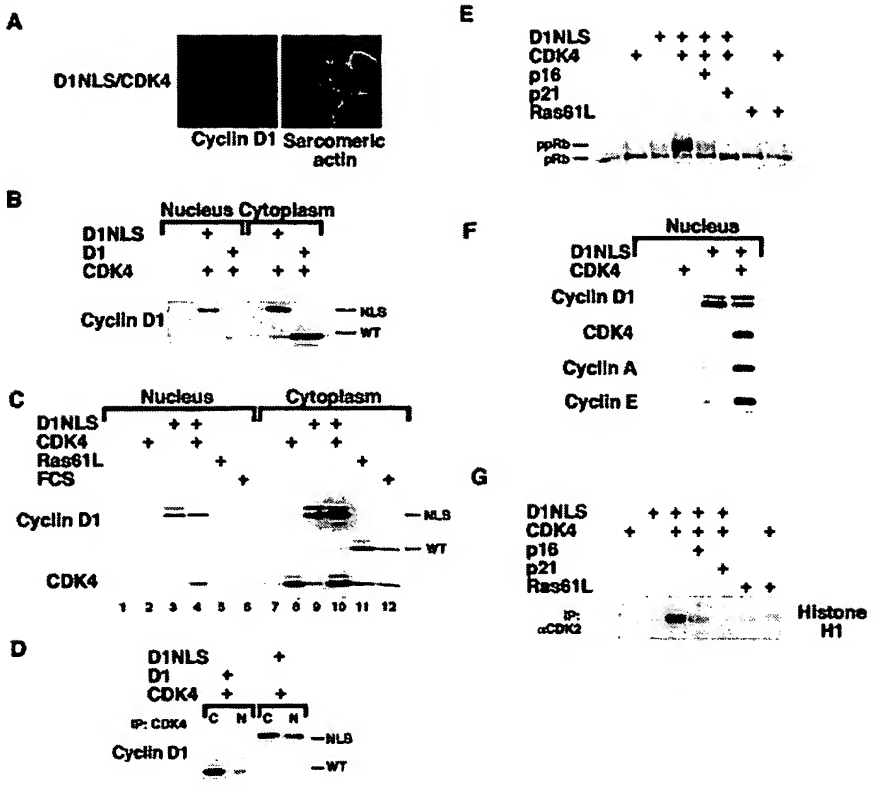

Figure 2. Nuclear import of cyclin D1/CDK4 induces Rb phosphorylation, CDK2 kinase activity, and promotes cell cycle progression in cardiomyocytes. Serum-starved cardiomyocytes were infected with the indicated viruses at MOI of 100 FFU/cell or stimulated with 10% FCS. Cells were harvested 48 hours after infection or 18 hours after FCS addition. A and B, D1NLS can localize in the nucleus in cardiomyocytes. A, Double immunofluorescent staining with anti-cyclin D1 (red) and anti-sarcomeric α-actin (green) as in Figure 1C. B, Nuclear and cytoplasmic extracts were assessed by Western blotting against anti-cyclin D1. C, Expression of D1NLS and CDK 4 in the nucleus. Nuclear and cytoplasmic extracts were assessed by Western blotting using the indicated antibodies. D, Association of D1NLS with CDK4 in the nucleus. The same extracts used in B were immunoprecipitated with anti-CDK4 antibody followed by Western blotting using anti-cyclin D1 antibody. E, Induction of Rb phosphorylation by D1NLS and CDK4. Whole-cell extracts were assessed by Western blotting using Rb antibody. pRb indicates underphosphorylated Rb; ppRb, hyperphosphorylated Rb. F, Induction of cyclin A and E expression by D1NLS and CDK4. Nuclear extracts were assessed by Western blotting using the indicated antibodies. G, Induction of CDK2 kinase activity by D1NLS and CDK4. Extracts were immunoprecipitated with anti-CDK2 antibody followed by histone H1 kinase assay as described.[11]

2D shows that D1NLS associated with CDK4 in the nucleus, whereas the wild-type cyclin D1/CDK4 complex was predominantly detected in the cytoplasm. Functionally, coexpression of D1NLS and CDK4 converted a majority of Rb into the hyperphosphorylated form (ppRb) (Figure 2E). Furthermore, D1NLS and CDK4 induced cyclin A and E expression and the CDK2 kinase activity, both of which are activated after Rb phosphorylation during the cell cycle (Figures 2F and 2G). Finally, that the Rb phosphorylation and CDK2 kinase activity depend on the kinase activity of D1NLS and CDK4 was confirmed by inhibition of these effects by coexpression of CKIs, $p16^{INK4a}$, and $p21^{Cip1}$ (Figures 2E and 2G).

The above results indicate that the nuclear import of cyclin D1/CDK4 can activate the Rb regulatory pathway that is required for cell cycle progression. Therefore, we tested using laser scanning cytometry (LSC) whether the nuclear import of cyclin D1/CDK4 could cause the reentry of postmitotic cardiomyocytes into the cell cycle. Serum-starved cardiomyocytes plated on coverslips were infected with adenoviruses or stimulated with FCS, then double-stained with propidium iodide (PI) and sarcomeric actin, a marker for cardiomyocytes. LSC analysis of sarcomeric actin–positive cells revealed that the overexpression of D1NLS and CDK4 caused a significant induction of cell cycle progression, as revealed by an increase of cells with S and G2/M DNA content, whereas FCS stimulation had little effect (Figure 3A). In addition, in the presence of 5% CS, D1NLS/CDK4 potentiated the activity, whereas coexpression of wild type cyclin D1 and CDK4 had no effect. Notably, D1NLS/CDK4 expression induced no apparent apoptosis as indicated by the absence of cells with sub-G1 DNA content in the infected cells. Furthermore, blocking the cells in G2/M phase with nocodazole revealed that ≈95% of the cells were in the S and G2/M fraction, whereas cells expressing wild-type cyclin D1 and CDK4 remained in the G1 fraction (Figure 3B). In addition, the G1 fraction reappeared after release from nocodazole in cells expressing D1NLS/CDK4. These results demonstrated that virtually all of the cells expressing D1NLS/CDK4 entered the cell cycle, leading to cell division.

To further confirm that the nuclear import of cyclin D1/CDK4 can induce mitosis and cell division of cardiomyocytes, we examined the morphology of cardiomyocytes expressing D1NLS/CDK4. LSC analysis allowed the DNA content of each cell in a defined microscopic field to be determined. Figure 4A shows many mitotic cells (Figure 4A, area vi; 15, 16, 18, 21) and cells immediately after mitosis (Figure 4A, area i; 4, 5, 7, 8, 9, 10), as indicated by high peaks of PI fluorescence, in sarcomeric actin–positive cardiomyocytes. In addition, daughter cells that appeared shortly after cell division resumed beating as parental cardiomyocytes (data not shown). Furthermore, the number of cardiomyocytes coinfected with these viruses tripled 5 days after infection (Figure 4B). We therefore conclude that the nuclear import of cyclin D1/CDK4 can induce the complete cell cycle progression of primary neonatal cardiomyocytes.

Finally, we examined the effects of nuclear import of cyclin D1/CDK4 on adult cardiomyocyte proliferation in

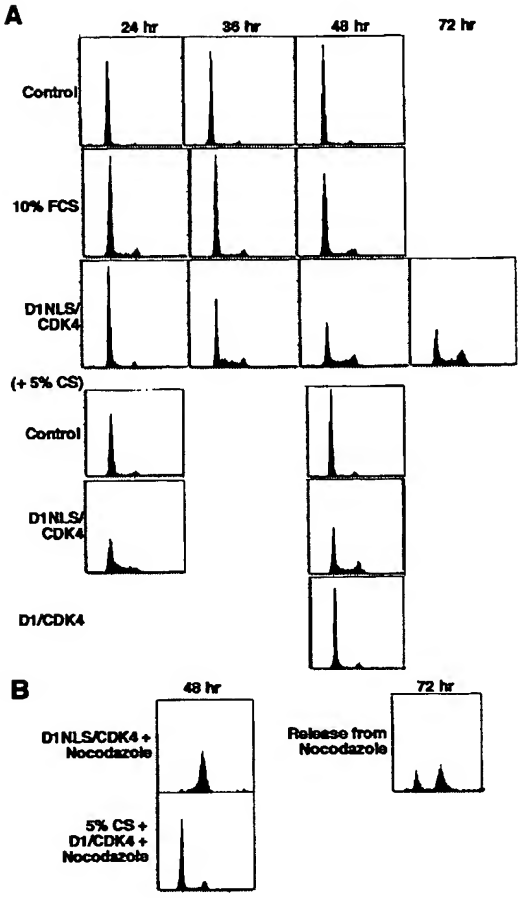

Figure 3. Nuclear import of cyclin D1/CDK4 promotes cell cycle progression in cardiomyocytes. Cells were infected with indicated viruses in the presence or absence of 5% CS. Cells were fixed at indicated times after infection and stained with anti-sarcomeric actin and FITC-conjugated anti-mouse antibodies, followed by propidium iodide containing RNase A. Cardiomyocyte cultures consisted of 97±1.5% FITC-positive cells that were confirmed by immunofluorescence microscopy. DNA content was analyzed on cell populations that expressed sarcomeric actin (FITC positive) using a laser scanning cytometer. Horizontal and vertical axes represent DNA content and cell number, respectively. A, Expression of cyclin D1/CDK4 in the nucleus leads cardiomyocytes to the cell cycle progression. B, Reappearance of G1 phase after G2/M block in cardiomyocytes infected with Ad-D1NLS and Ad-CDK4. Nocodazole (50 ng/mL) was added 24 hours after infection, after which cells were cultured for 24 hours and then fixed or released from G2/M block by washing and incubating in serum-free media for another 24 hours.

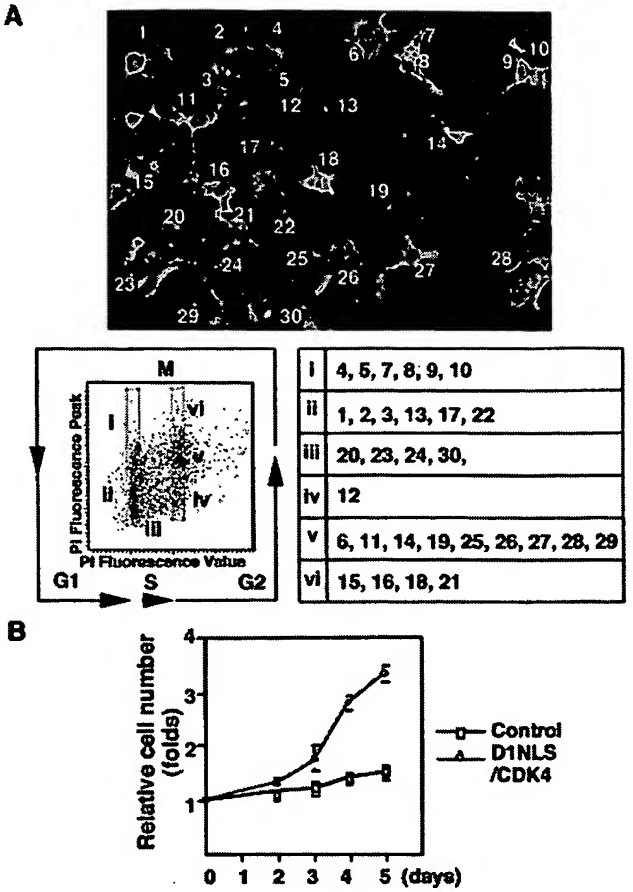

Figure 4. Nuclear import of cyclin D1/CDK4 promotes cell division of cardiomyocytes. A, Confocal image of cardiomyocytes infected with Ad-D1NLS and Ad-CDK4. Cells fixed 48 hours after infection were stained with sarcomeric actin (green) and PI (red) as in Figure 3, after which cells were analyzed by LSC (bottom) and then morphology was visualized using a confocal laser microscope (FV500) (top). Numbers indicate cycle positions of cells analyzed by LSC. i through vi indicate areas determined by total value and peak of PI fluorescence. B, D1NLS/CDK4 increased numbers of cardiomyocytes. Cells were counted at the indicated days after infection.

vivo. We injected the cyclin D1NLS virus or cyclin D1WT virus, together with the CDK4 virus, into the apical regions of rat hearts and fixed 4 days after infection. In addition, to identify the infected area, Ad-LacZ was coinfected with these viruses. We used hearts infected with only the LacZ virus for controls. Because the Ki-67 nuclear protein is expressed in proliferating cells in all phases of the cell cycle and associated with cell division,[29] we first stained infected heart tissues with anti-Ki-67 antibody, followed by staining with anti-β-gal and sarcomeric actin antibodies to identify infected cardiomyocytes. Ki-67 expression was detected in a number of nuclei in β-gal–positive cardiomyocytes and nonmyocytes, such as interstitial cells, in the regions coinjected with the cyclin D1NLS and CDK4 viruses (Figures 5A i and j, and 5B a). In contrast, the expression was not detected in cardiomyocytes infected only with the LacZ virus, or with the wild-type cyclin D1/CDK4 viruses (Figure 5A d and e; data not shown). Although recent studies have shown that a small population of cardiomyocytes that appear not to be terminally differentiated exists in the adult heart, the morphology of the Ki-67–positive cardiomyocytes was indistinguishable from that of adjacent fully differentiated cardiomyocytes in the uninfected regions.

Previous experiments have shown that adenoviral delivery of E2F1, a downstream effector of the cyclin D1 pathway, leads to an apoptotic response in adult cardiomyocytes.[30] Therefore, we examined the viability of Ki-67–positive cardiomyocytes produced by the infection of the D1NLS and CDK4 viruses. We performed in situ TUNEL staining to detect apoptotic cells in the infected regions. Figure 5B shows that, however, no TUNEL-positive nuclei was detected in the Ki-67–positive cardiomyocytes. Furthermore, nuclear staining with DAPI revealed that nuclear morphology of these cells retained normal shapes and no

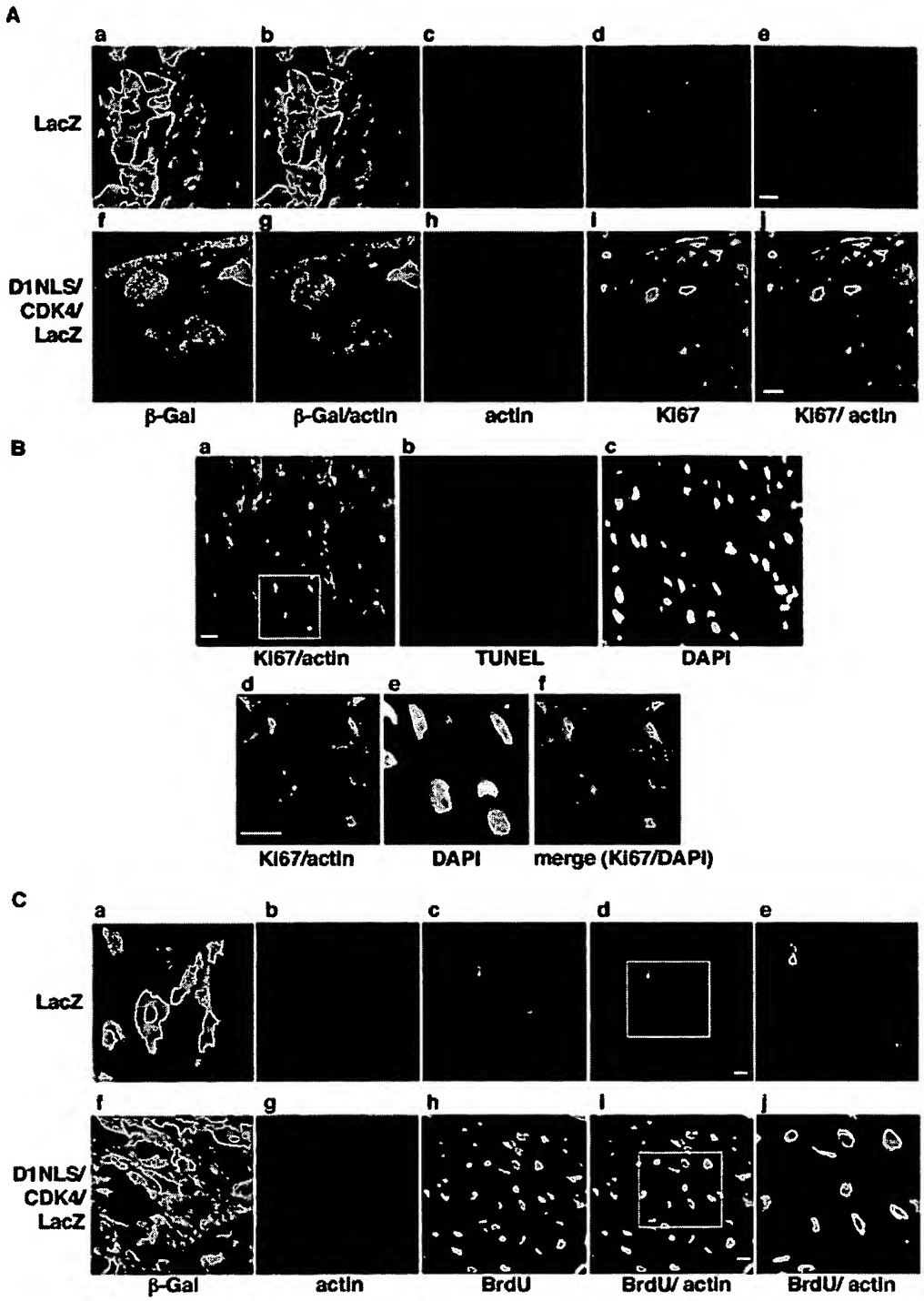

Figure 5. D1NLS/CDK4 promoted cell cycle entry in adult cardiomyocytes in vivo. A, Induction of Ki-67 by D1NLS/CDK4. Adult hearts were infected with indicated viruses, fixed, and stained with β-gal (green), sarcomeric actin (red), and Ki-67 (green). Bar=10 μm. B, D1NLS/CDK4 did not induce apoptotic response in cardiomyocytes. a through c, Sections of heart infected with the D1NLS and CDK4 viruses were subjected to in situ TUNEL staining (green), followed by staining with Ki-67 (green), sarcomeric actin (red), and DAPI (white). Bar=10 μm. d through f, Enlarged images of area marked by a rectangle in a are shown. Ki-67, green; sarcomeric actin, red; DAPI, white (e) or red (f). Bar=10 μm. C, Induction of BrdU incorporation by D1NLS/CDK4. Adult hearts were infected with indicated viruses and subjected to BrdU incorporation assay. Enlarged images of area marked by a rectangle (d and i) are shown in e and j. β-gal, green; sarcomeric actin, red; BrdU, green. Bar=10 μm.

blebbing and shrunken nuclei, which are characteristic in apoptotic cells, were detected. In contrast, we observed various patterns of Ki-67 subnuclear localization, which changes drastically during the cell cycle,[29] in cells infected with the D1NLS and CDK4 viruses.

Finally, to directly assess cell cycle reentry of adult cardiomyocytes by D1NLS and CDK4, we examined DNA synthesis of infected cardiomyocytes. Infected rats were injected BrdU every 12 hours and subjected to BrdU incorporation assay 4 days after infection. Figure 5C shows that a number of BrdU-positive

cardiomyocytes were detected in the regions infected with the D1NLS/CDK4 viruses. In contrast, only sarcomeric α-actin negative–cells were stained in areas infected with the control LacZ virus. Taken together, these results indicate that the nuclear import of cyclin D1/CDK4 could promote cell cycle entry of adult cardiomyocytes in vivo.

Discussion

In the present study, we show that the nuclear import of the cyclin D1/CDK4 complex is tightly prevented in postmitotic cardiomyocytes. Furthermore, overcoming of this inhibitory mechanism is sufficient to induce cell cycle progression leading to cell division of neonatal cardiomyocytes. In addition, the daughter cells after cell division resumed beating and expressed sarcomeric actin, a marker for cardiomyocytes. The critical role of cyclin D1–dependent kinase activity in cell cycle progression of quiescence or terminally differentiated cells is supported by the recent studies using other types of cells, albeit the cyclin D1 activity is not sufficient to promote cell division in these cases.[31–33] In this regard, previous studies have shown that activation of the Rb pathway by forced expression of adenovirus E1A or E2F1, a downstream target of Rb, can induce DNA synthesis but resulted in apoptotic cell death in cardiomyocytes.[34,35] Thus, our findings indicate that the nucleocytoplasmic transport machinery of cyclin D1 plays a critical role for determining proliferative capacity of cardiomyocytes.

The precise mechanism preventing cyclin D1 nuclear accumulation remains unclear. Nucleocytoplasmic transport pathways of proteins are selectively regulated by various transport receptors and signals, such as NLSs and nuclear export signals (NESs), and are involved in various cell functions including cell cycle control.[36,37] D-type cyclins and CDK4 lack consensus NLSs, and $p21^{Cip1}$ and $p27^{Kip1}$, which contain NLSs, promote the assembly and nuclear localization of the cyclinD1/CDK4 complex.[9,27,28] However, studies on primary mouse embryonic fibroblasts (MEFs) lacking both $p21^{Cip1}$ and $p27^{Kip1}$ have demonstrated that cyclin D1 by itself is able to localize in the nucleus without detectable assembly with CDK4. Furthermore, these MEFs still retain some cyclin D–dependent kinase activity and exhibit no apparent effects on the cell cycle.[27] In postmitotic cardiomyocytes, we show that the cyclin D1/CDK4 complex was formed but remained in the cytoplasm (Figure 2D). In addition, the cytoplasmic cyclin D1/CDK4 complex associated with $p21^{Cip1}$ and ectopic expression of $p21^{Cip1}$ or $p27^{Kip1}$ did not promote nuclear localization of cyclin D1/CKD4 (unpublished data, 2002). On the other hand, the subcellular localization of cyclin D1 is also regulated by the phosphorylation of cyclin D1 at threonine286 by glycogen synthase kinase 3β (GSK3β) that promotes the nuclear export and degradation of cyclin D1 during S phase.[24–26,38] Inhibition of endogenous GSK3β activity by an adenovirus encoding a dominant negative mutant of GSK3β (GSKDN) resulted in some increase in cyclin D1 accumulation in the nucleus (unpublished data). Therefore, it is possible that the GSK3β -mediated nuclear export of cyclin D1 may contribute, at least to some extent, to the cytoplasmic sequestration of cyclin D1 in postmitotic cardiomyocytes. Additionally, although addition of efficient NLSs promotes nuclear localization of cyclin D1, the majority of D1NLS protein was still detected in the cytoplasm (Figures 2B and 2C). This may also support the notion that postmitotic cardiomyocytes have machinery positively preventing cyclin D1 nuclear localization, resulting in significant sequestration of D1NLS protein in the cytoplasm.

The inhibition of cyclin D1 nuclear accumulation is likely to be a critical barrier for maintaining cardiomyocytes tightly in the postmitotic state. Indeed, overexpression of wild-type cyclin D1 and CDK4 in reversibly quiescence fibroblasts, as well as proliferating fetal cardiomyocytes, caused nuclear accumulation of these proteins (M. Tamamori-Adachi, P. Sumrejkanchanakij, M.-A. Ikeda, unpublished observations, 2002) and has been shown to induce DNA synthesis.[31–33] Thus, prevention of their nuclear import is unlikely to be a common feature in cells reversibly arrested in G0 phase. Rather, it appears to be a characteristic of postmitotic cardiomyocytes, which may play a critical role in switching from a proliferating state to a terminally differentiated state in cardiomyocytes and, possibly, in other terminally differentiated cells. A recent study reported that cyclin D1 overexpression can induce the reentry into the cell cycle from the terminally differentiated state in some types of established cell lines.[33] We show, however, that ectopically expressed cyclin D1 was unable to localize in the nucleus and to induce cell cycle progression in neonatal cardiomyocytes. Thus, it is likely that postmitotic cardiomyocytes have a unique property in the nucleocytoplasmic transport of cyclin D1.

Finally, we show that the nuclear import of cyclin D1/CDK4 induces the cell cycle reentry of a number of cardiomyocytes in the adult heart as demonstrated by expression of the Ki-67 nuclear antigen and BrdU, markers associated with cell cycle entry. Furthermore, we did not detect apoptotic induction in these cardiomyocytes. Although recent evidence has demonstrated that the adult heart contains cardiomyocytes that have proliferative capacity,[4] Ki-67 expression was only detected in a small population of myocytes in regions adjacent to the infracts. Therefore, our results suggest that the nuclear import of cyclin D1/CDK4 may potentiate the ability of cardiomyocytes that retain proliferative capacity. It is also possible that their nuclear import might promote the cell cycle reentry of a population of cardiomyocytes that otherwise do not enter into the cell cycle in the adult heart. Nevertheless, previous experiments using transgenic mice carrying wild-type cyclin D1 driven by α-cardiac myosin heavy chain (MHC) promoter have shown that deregulated wild-type cyclin D1 expression causes an increase in cardiomyocyte number and cardiomyocyte DNA synthesis in the adult heart.[39] In this study, however, we show that transient expression of cyclin D1 did not promote cell cycle progression in both neonatal and adult cardiomyocytes. Given that the α-cardiac MHC gene begins to express during late stages of embryogenesis when cardiomyocytes have proliferative capacity and allow cyclin D1 nuclear accumulation (Figure 1D), it is possible that deregulated wild-type cyclin D1 expression might prevent cell cycle withdrawal of terminally differentiated cardiomyocytes, which normally occurs soon after birth. Alternatively, we cannot rule out the possibility that constitutive cyclin D1 expression might cause cell cycle

reentry of the adult heart for long period of time in the transgenic model. Investigation of the molecular mechanism preventing cyclin D1 nuclear import in postmitotic cardiomyocytes will provide findings important to the development of strategies for regenerating cardiomyocytes. Although the engraftment of cells derived from embryonic or bone marrow stem cells may be useful for regenerating the functional myocyte mass in the adult heart,[5–7] we believe that our results provide an important step toward the development of an alternative therapeutic application.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, the Japan Society for the Promotion of Science, and a grant from the Atsuko Ouchi Memorial Fund. We thank Joseph. R. Nevins, Jeffrey. H. Albrecht, and Sander. van den Heuvel for materials, and Mieko Goto and Shiho Hashimoto for excellent technical assistance. We are also grateful to and Yoshimasa. Kiyomatsu for LSC analysis and Joseph. R. Nevins for critical reading of the manuscript.

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